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Do tsetse flies only feed on blood?

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ABSTRACT

Tsetse flies (*Diptera*: *Glossinidae*) are the vectors of trypanosomes causing sleeping sickness in humans, and nagana (animal trypanosomosis) in domestic animals, in Subsaharan Africa. They have been described as being strictly hematophagous, and transmission of trypanosomes occurs when they feed on a human or an animal. There have been indications however in old papers that tsetse may have the ability to digest sugar.

Here we show that hungry tsetse (*Glossina palpalis gambiensis*) in the lab do feed on water and on water with sugar when no blood is available, and we also show that wild tsetse have detectable sugar residues. We showed in laboratory conditions that at a low concentration (0.1%) or provided occasionally (0.1%, 0.5%, 1%), glucose had no significant impact on female longevity and fecundity. However, regular provision of water with 1% glucose increased the mortality and reduced the fecundity of female *G. p. gambiensis*. The proportion of wild tsetse caught by traps, which have detectable sugar residue in their midgut varied between 5 and 10% according to species ($p < 10^{-3}$) and sex, with more females being found with sugar residues than males ($p < 10^{-3}$). We also observed a higher frequency of sugar residues in the dry season than in the rainy season ($p < 10^{3}$). The infection status did not affect the frequency of sugar residues found ($p = 0.65$), neither did age ($p = 0.23$).

These observations represent a fundamental change in our knowledge of this insect vector. They open the way for further research on the field to know more on tsetse feeding behavior regarding other sources of meal than blood, in particular with plants, and may constitute future new means of controlling this vector of neglected tropical diseases.

1. Introduction

African trypanosomiases affect humans (HAT: human African trypanosomiasis, also called sleeping sickness) and livestock (AAT: animal African trypanosomosis, or nagana) throughout sub-Saharan Africa with an estimated 50 million people at risk of infection. WHO and the African Union declared elimination to be the objective (Kabayo, 2002; WHO, 2012). Rearing livestock in endemic areas is difficult or even impossible, and results in economic losses in agricultural output of several billion US dollars per year (Vreysen et al., 2013). Human infections are fatal if untreated, and tools for disease control are limited. So far, it has been impossible to develop vaccines, and current trypanocidal drug treatments have either undesirable side effects or are difficult to administer (Simarro et al., 2012). The biological vector of human African trypanosomiasis and nagana in livestock is the tsetse fly (*Diptera*: *Glossinidae*). The reduction or elimination of tsetse populations is an effective method for controlling trypanosomiasis that requires a thorough knowledge of tsetse biology and ecology.

Tsetse flies are key representatives of the dipteran clade *Calyptratae*, a majority of calyptrate species being blood feeders of biomedical importance. Tsetse flies are specific to the *Glossinidae* family which only contains the *Glossina* genus, and currently has 31 recognized taxa (species and subspecies). Members of the super family *Hippoboscidea* and of the *Glossinidae* family are defined by hematophagy in both sexes, their ability to nourish intrauterine offspring from glandular secretions and give birth to fully developed larvae (obligate adenotrophic viviparity) (Itard and Cuisance, 2003; Hargrove, 2004). Tsetse flies live significantly longer than other vector insects, which compensates for their slow rate of reproduction.

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It has been thought for long that *Glossina spp*. differed from other blood feeding insects including mosquitoes, which consume plant nectar to supplement their diet. Tsetse use a proline–alanine shuttle system for energy distribution and triglycerides/diglycerides for storage in body fat and milk secretions as a probable result of evolutionary adaptation to hematophagy (Bursell, 1975, 1963). This suggests that sugar may not be required for basic tsetse metabolism, at least for energy. However, there have been some controversies on this issue that still continue today, since little to no sugar or glycogen was found in tsetse by some authors (Ribeiro et al., 2010), whereas other authors reported large amounts of glycogen in the proventriculus of tsetse (D'Costa et al., 1973), and in another study, tsetse were found to be able to metabolize injected glucose (Nayar and Van Handel, 1972).

Hence a very simple question arose: if tsetse, which have always been described as obligatory bloodfeeders, cannot find blood in their habitat for any reason, would they be able to feed on another source of meal? And would it be possible that they would be able to include sugar in their meals, compared to the numerous other dipteran vectors that do so? Further to the academic interest of understanding how this insect vector behaves, answering this question may provide new opportunities to improve the control of this major vector of a deadly neglected tropical disease. To answer this question, we conducted laboratory experiments and a field study, both of which showed that tsetse are able to feed on sugar and water in the lab, and are found with sugar residues in the wild. In addition, although permanent feeding with high concentrations of sugar appeared to be toxic to tsetse, sugar added to their diet either occasionally or at low concentrations did not affect their mortality and fecundity.

2. Material and methods

2.1. Laboratory experiments

2.1.1. Effect of giving sugar and water to starved teneral tsetse at their first meal

At the tsetse insectarium in Montpellier, at 25 °C and 85% relative humidity, teneral males and females *Glossina palpalis gambiensis* in Roubaud cages were given the possibility to take water, and water with 5% glucose, as their first meal post-emergence. They were either given the meal through a silicone membrane at J1 or J2 post-emergence. Water only, and water with 5% glucose, were colored with a food coloring agent, in order to make it more visible. The number of tsetse having taken water only, or water with glucose, was then assessed by dissecting them and looking at their gut content.

2.1.2. Sugar detection and digestion kinetics

Another aim of the experience was to check if sugar added to tsetse blood meal was detectable in the midgut of tsetse using the cold anthrone test (Fig. S1) (Van Handel, 1972), and to monitor the dynamics of this detection. After incubation at room temperature for 60 min in the presence of sugar residues in the midgut, the yellowish anthrone reagent turned green or dark blue. Laboratory experiments were conducted in the CIRDES tsetse rearing facility, at a temperature of 25 °C and 75–80% relative humidity. Individuals of *G. p. gambiensis* and *Glossina morsitans submorsitans* colonies (both originating from CIRDES insectary) were selected immediately after emergence and maintained in starvation conditions in Roubaud cages at a maximum density of 40 flies per cage for two days, to increase the probability of their feeding once offered a meal. On the third day, the flies were fed with cattle blood (originating from the slaughter-house of Bobo-Dioulasso) through a synthetic silicone membrane using the standard method of feeding in the insectarium (Kabore and Bauer, 1984). The blood was previously defibrinated and then sterilized by irradiation using gamma rays to prevent potential bacterial contamination.

Three hundred male *G. p. gambiensis* and 210 female *G. m. submorsitans* were used in this experiment. The flies in the control group were fed with natural blood. The five treated groups were fed with blood in which increasing doses of glucose were added: 1 g glucose/100 ml blood (1%, or 55 mmol L^{-1}), 2 g glucose/100 ml blood (2%), 3 g glucose/100 ml blood (3%), 4 g glucose/100 ml blood (4%), and 5 g glucose/100 ml blood (5%) respectively. Flies in the different groups were fed their respective type of meal only once, after which the engorged tsetse were dissected in batches of 10 flies per type of meal at different periods: T0 (immediately after feeding), T4 (4 h after feeding), T8 (8 h), T24 (24 h), T48 (48 h), T72 (72 h) and T96 (96 h). Each fly's midgut was collected and placed individually in a 1.5 ml Eppendorf tube and 0.5 ml of anthrone reagent was added for the detection of sugar.

2.1.3. Effect of consuming sugar on tsetse survival and fecundity

The effect of four different feeding regimes on the survival and fecundity of females of *G. p. gambiensis* was tested. As their first meal, four batches of flies were fed either blood alone (control), blood with 0.1% glucose (0.1 g glucose per 100 ml of blood), 0.5%, and 1% glucose respectively. Three-day old females were mated with seven-day old males (using the standard procedure at the tsetse rearing facility) and kept together for a week before being separated. Females were then monitored and different parameters (mortality, number of pupae per female and pupal weight) were recorded daily. In all the experiments, the flies were monitored for 60 days. To ensure the good quality and homogeneity of the blood, the meals were prepared every three days and stored at 4 °C.

2.1.3.1. Experiment 1: Effect of consuming sugar once or twice. The aim of this study was to test the effect of a single blood meal complemented with sugar before mating alone, and of a second one post mating on longevity and fecundity. To this end, three concentrations of glucose (0.1%, 0.5% and 1%) added to blood were compared to the control (blood alone). Forty females were fed once before and once after mating with the four types of meals and thereafter with blood only. Two repetitions were conducted in each case.

2.1.3.2. Experiment 2: Effect of multiple sugar consumption. In this experiment, we investigated the effect of regular meals containing glucose added to blood. Two concentrations (0.1% and 1%) were tested. Forty flies were fed daily with each concentration while control flies received standard blood. Five repetitions were conducted for each treatment.

2.1.4. Negative control group

To validate the positive results of the anthrone test, we conducted an experiment on negative controls. For this experiment, 200 flies (100 *G. p. gambiensis* and 100 *G. m. submorsitans*, with 50 males and 50 females in each case) were fed only once using standard blood. Each day, 20 flies of each species were dissected and subjected to the anthrone test, then monitored for five days.

2.2. Field observations

The main aim of this preliminary field study was to determine if sugar residues could be detected in wild tsetse, and if there was any effect of season, tsetse species, age, and infection with trypanosomes on the number of tsetse found with these sugar residues.

Field studies were conducted in the area of Folonzo, southern Burkina Faso. The presence of tsetse species and their biotope are very well documented in this area (Courtin et al., 2009; Rayaisse et al., 2009; Salou et al., 2012). Several tsetse species live there in sympatry: *G. p. gambiensis*, *Glossina tachinoides* (both belonging to the same *Nemorhina* subgenus), *G. m. submorsitans* (*Glossina* subgenus) and *Glossina medicorum* (*Austenina* subgenus).

Ecologically, *G. medicorum* is a forest species, whereas *G. p. gambiensis* and *G. tachinoides* are riverine species and live mainly in the gallery forest bordering the river. These species, particularly *G. tachinoides* which is more xerophilic, also penetrate the bordering

savannah to look for hosts, where *G. m. submorsitans*, a savannah species, is predominant. Wild mammals and reptiles are commonly found in the area and, in addition to humans, are the main source of blood meals for riverine tsetse.

Biconical traps (Challier and Laveissière, 1973) were set up along the Comoe River in the dry season in February and April 2014 and in the wet season in June 2014. The cages containing the flies were collected every 24 h to be sure that tsetse were still alive. Once collected, the cages containing tsetse were kept in a humidified container and transferred to the field team base where they were identified according to species and sex, dissected, and the midgut used for the sugar detection with anthrone in the same way as in the lab experiments. The ovary of the same flies was also dissected to determine the physiological age using binoculars. The proboscis was observed under a microscope (*400 magnification) to look for trypanosomes.

2.3. Statistics

2.3.1. Laboratory study

To compare the probability of survival between batches, the impact of the treatment regime was analyzed using a generalized linear mixed model with a Poisson distribution. The treatment regime was used as the unique fixed effect and the number of the repetition as a random effect. The observation censoring was accounted for using an offset term based on the logarithm of days at risk (sum of the number of days the flies survived during the observation period). The same analysis was conducted for fecundity, defined here as the number of pupae produced by the cohort over the observation period (and corrected for survival).

Finally, the weight of the pupae (Fig. S2) was analyzed using a generalized linear mixed model with a normal distribution, using the treatment regime and the age of the flies as fixed effects, and the number of the repetition as a random effect.

2.3.2. Field study

The proportion of flies that had detectable sugar residues in their midgut was analyzed using generalized linear mixed binomial models fit by maximum likelihood (Laird and Ware, 1982). The tsetse species, sex, and season were used as fixed effects and the position of the trap as a random effect to account for possible spatial variations in sugar availability.

The same analysis was conducted on the subsample of dissected flies whose rate of infection by trypanosomes was known, except that the infection status was added as a fixed effect, and the season was not considered, since this study was only conducted in the dry season. These two variables, which were also only available for the dry season, were added as fixed effects in the subsample of dissected females for which the presence of a larva in the uterus and the physiological age were known.

In all the analyses, the best model was considered to be the one with the lowest corrected Akaike information criterion (AICc) (Burnham and Anderson, 2002; Hurvich and Tsai, 1995). R software (R Core Team, 2015) was used for statistical analyses, with the lme4 package for the linear mixed-effect model (Bates et al., 2011) and the MuMin package for the implementation of the AICc (Burnham and Anderson, 2002).

3. Results

3.1. Laboratory results in Montpellier

Out of 115 males *G. p. gambiensis* that were given the possibility to have their first meal (at J1 and J2 post-emergence) consisting of water only, 51 (44%) took water and 64 (56%) did not, vs 30 (29%) and 75 (71%) out of the 105 males that were given water with 5% sugar. Out of the 93 females that were offered water, 49 (53%) fed on it and 44 (47%) not, vs 50 (52%) and 46 (48%) out of 96 females that were offered water with 5% glucose. This first experiment showed that tsetse can feed on other source than blood. A video of a teneral female *G. p. gambiensis* feeding on water with 5% glucose colored in blue is available online.[1]

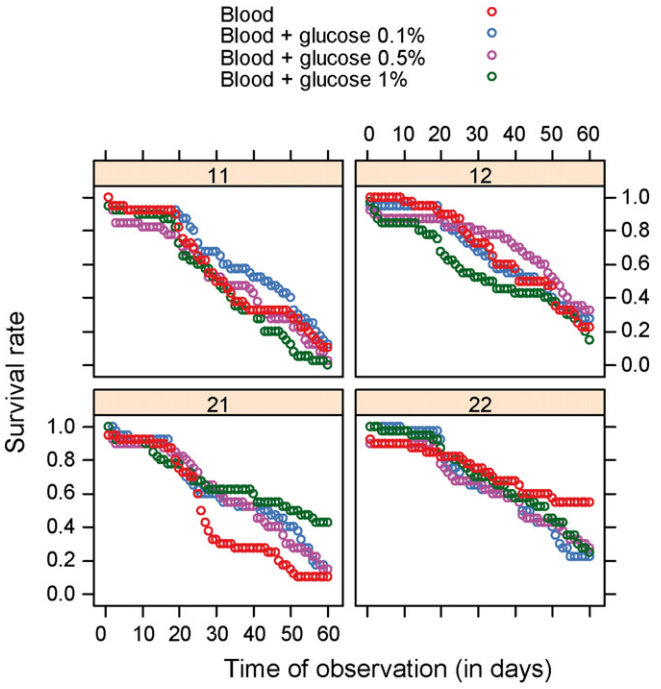

Fig. 1. Survival rates of tsetse fed with two blood meals containing different concentrations of glucose. Females *G. p. gambiensis* were maintained in starvation in Roubaud cages at a maximum density of 40 flies per cage for two days to increase their probability of feeding once they were presented with food. On the third day, they were fed different types of meals through a synthetic silicone membrane. Forty flies were fed one of the four types of meal (indicated by the colored points in each panel) once before and once after mating and thereafter with blood only. Panels 11 and 12 correspond to two repeats run in the same time during the first period and 21 and 22 to the two repeats of the second period. All treatment regimens were applied in each of these repeats. Climatic conditions in the laboratory were 25 °C temperature and 75–80% relative humidity. In all the experiments, tsetse mortality was checked daily for 60 days.

3.2. Laboratory results in Bobo-Dioulasso

When 3% to 5% glucose was added to blood meals used to feed tsetse in the lab, the anthrone test (Figure S1) was able to detect it in both *G. p. gambiensis* (males) and *G. m. submorsitans* (females) up to 96 h post ingestion. At 1%, it was detectable in *G. p. gambiensis* only up to 4 h post ingestion and at 2%, up to 8 h. When no glucose was added to the blood, all the anthrone tests (200) were negative, as stated by the "negative controls" experience. In addition, 48 h after feeding, we observed about 40% mortality in females of *G. m. submorsitans* fed at concentrations of 3% and 5%. In contrast, no mortality was observed in *G. p. gambiensis* fed with different concentrations of glucose at any time during the course of the experiment.

When tsetse were fed with only two meals with different concentrations of sugar (the other meals being natural blood), the feeding regime did not affect tsetse survival (Fig. 1 and Table S1). Neither did it affect fecundity, except for a marginal positive effect detected when the blood was complemented with 1% glucose ($p = 0.083$, Table S2). Finally, the feeding regime had no impact on the weight of the pupae ($p = 0.17$; Figure S2), although their weight decreased with the age of the fly ($p < 10^{-3}$).

However, when female tsetse were fed daily with added sugar, a 1% concentration of sugar significantly increased mortality in all

[1] https://www.youtube.com/channel/UCJdINlL7VyYvnck5eXD0bow?feature=em-upload_owner.

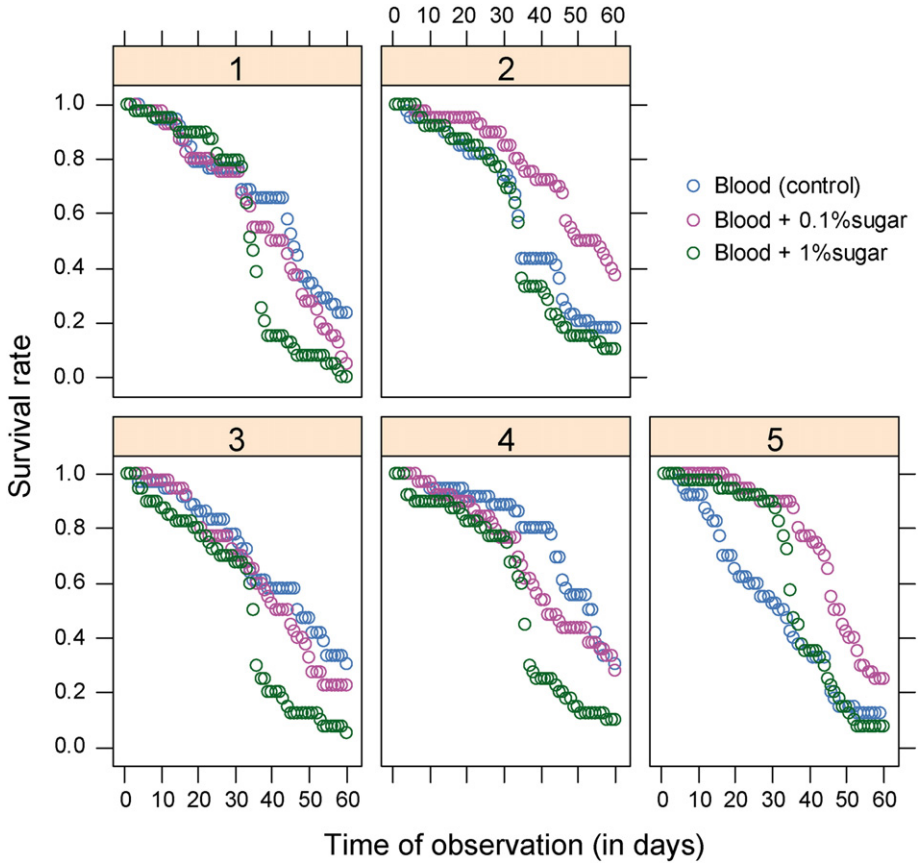

Fig. 2. Survival rates of female *G. p. gambiensis* in the laboratory. Here females were fed continuously with blood containing 0.1% or 1% of sugar (five repeats). Flies were kept in the same climatic conditions as in Fig. 1.

replications (Fig. 2 and Table S3, $p = 0.004$). A 0.1% concentration of sugar had no impact on fecundity ($p = 0.4$) whereas a 1% concentration significantly reduced fecundity ($p = 0.02$, Table S4). Like in the first experiment, the type of treatment had no impact on the weight of the pupae ($p = 0.12$), which again decreased with the age of the fly ($p < 10^{-3}$).

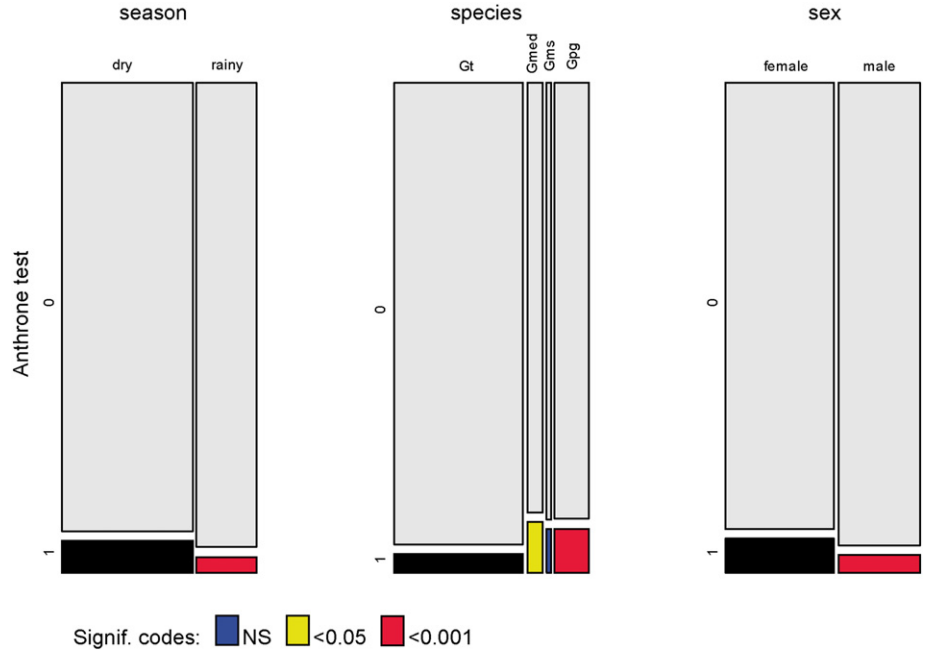

Fig. 3. Proportion of flies with positive anthrone tests according to season, species, and sex. The p-value corresponds to the fixed effects in the mixed generalized model (see text for explanation) and is given for comparison to the reference category of the variable (black). The size of the boxes are proportional to the sampling effort (total of 1430 flies tested). Wild flies were captured at 24 h intervals with biconical traps placed on the shore of the River Comoe, southern Burkina Faso, in the dry (February to April 2014) and wet season (June 2014).

3.3. Field results

A total of 1430 tsetse comprising 1010 *G. tachinoides*, 263 *G. p. gambiensis*, 124 *G. medicorum* and 33 *G. m. submorsitans* were collected at Folonzo, south-western Burkina Faso in 2014. Approximately 6% of them were positive to the anthrone test, with more positives in both *G. medicorum* and *G. p. gambiensis* than *G. tachinoides* and *G. m. submorsitans* ($p < 10^{-3}$). Females were more frequently positive to the anthrone test than males ($p < 10^{-3}$, Fig. 3). Out of the 321 dissected females, only 1.8% of the females with an empty uterus had sugar residues vs. 11.7% females with a larvae, but the difference was not significant ($p = 0.1$). The infection status did not affect the frequency of sugar residues in the midgut ($p = 0.65$), neither did age ($p = 0.23$). We observed a higher frequency of sugar residues in the midgut in the dry season than the rainy season (Fig. 3, $p < 10^{-3}$).

4. Discussion

Here, we show for the first time that in the lab, 30 to 50% of tsetse which are hungry do feed on water containing glucose or water only, if no blood is offered to them. We also find that high concentrations of glucose (more than 3% in the blood meal) caused acute mortality in *G. m. submorsitans*, but that glucose occasionally added to the blood meal had no adverse effect on tsetse longevity and fecundity. We observed in the field that various species belonging to the three main groups of *Glossina* have detectable sugar residues in their midgut.

What suggests the experience of hungry tsetse that fed on water and on water with glucose is that, if for unknown reasons tsetse are not able to find a blood source, they may well feed on what they can find, water or sugar for instance. This result is totally different from previous experiments showing that they can feed on other mediums than blood (Schoni et al., 1982). This was observed here for *G. p. gambiensis*, which is well known for its opportunistic feeding behavior regarding host choice, but the question remains for other tsetse species.

Does a positive result in the anthrone mean that tsetse did consume sugar and a negative reaction that they did not? The anthrone test detects sugar, in particular plant fructose (Van Handel, 1972), and the test has been used in other *Diptera* to answer exactly the same kind of question (see for instance (Gouagna et al., 2010) for *Anopheles gambiae*). Our experiments showed that 3% of glucose added to blood meal was detectable up to four days post-feeding. We also demonstrated unequivocally that the results of the anthrone test were consistently negative when no glucose was added to blood meals. These blood meals in Bobo-Dioulasso come from the local slaughterhouse and usually consist of natural blood from cattle, pigs and horses. This shows that the test is able to detect the glucose that was added to blood, because of its high concentrations, ranging from 55 to 275 mmol L^{-1}, whereas it was negative for physiological concentrations (3, 5 to 6,1 mmol/L in mammals). Thus, the anthrone test on tsetse midguts does not detect sugar at physiological concentrations of mammalian blood.

In the laboratory, we showed that *G. m. submorsitans* tolerates glucose much less than the riverine *G. p. gambiensis*. What also became apparent was that glucose consumed occasionally (i.e. when added to the blood only once or twice) had no effect or a marginally positive effect on longevity and fecundity, whereas consumed on a regular basis, it was toxic. The only negative effect would come from the weight of the pupae, which decreased with age, whereas the opposite was reported, e.g. the weight of pupae may increase when female become older (Clutton-Brock and Langley, 1997). The fact that glucose is not needed for tsetse, basic metabolism has also been confirmed by the recently published genome of *Glossina morsitans* showing a marked reduction in genes associated with carbohydrate metabolism, because tsetse use a proline–alanine shuttle system for energy distribution and triglycerides/diglycerides for storage in body fat and milk secretions (International Glossina Genome Initiative, 2014). But our observations also suggests that "all tsetse are not equal" with respect to sugar, since *G. p. gambiensis* appears to tolerate sugar much better than *G. m. submorsitans*. Publication of genomes of other tsetse species including those of the *palpalis* group in the coming months may provide new information on this topic (G. Attardo, pers. com.).

The 6% of field-caught tsetse whose midgut contained sugar residues, suggesting that they consumed sugar in the preceding 96 h, adds to the lab experience point in suggesting that tsetse may take other source of meals than blood only, if blood is not available for any reason. Such reasons may include hot unavailability at given periods, or decrease in flight availability for some individuals. It also raises many questions since we do not know so far from what sources these sugar residues come from, and at what frequency they are taken.

We also observed a significant difference among species with an increased frequency of sugar residues during the dry season, in both *G. p. gambiensis* and *G. medicorum*, two species that are much less xerotolerant than *G. m. submorsitans* and *G. tachinoides*. This observation is also in line with our lab observations suggesting that *G. p. gambiensis* tolerates sugar much better than *G. m. submorsitans*. Although not significant, the trend suggesting that sugar is found more frequently in female tsetse carrying a larva is also in line with this hypothesis.

We found no correlation between infection by trypanosomes in the tsetse caught, and the presence of sugar residues in our study. However an underestimation of positive cases of infection is possible because only the proboscis was observed for finding trypanosomes, since the gut had been collected for the anthrone test. It may be interesting to go further on these tsetse–trypanosome interactions having in mind this new data on tsetse feeding behavior, and keeping in mind that trypanosomes are also able to use unusual metabolic pathways linked to glucose and amino acid degradation (Bringaud et al., 2015).

Hence, apart from a fundamental change in our understanding of the biology of tsetse flies, these results pose numerous questions. Answering these questions might allow a better understanding of tsetse biology, the epidemiology of trypanosomes and even impact on vector control. These results are all the more intriguing than female tsetse were shown to be attracted by plants volatiles extracted from leaves and flowers of plants, for instance *Lantana camara* (Syed and Guerin, 2004).

Finally, we hope that our results will pave the way to a response to this comment of P.A. Buxton (1955) "There is not the least doubt that blood, without any other diet, is normally sufficient for these insects… The question however remains whether when water is available, the wild *Glossina* ever drinks, which might be of great importance in assisting its survival. The evidence is curiously indefinite."

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.meegid.2015.09.016.

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